

The influence of the mode of administration in the dissemination of three coliphages in chickens

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ABSTRACT *Escherichia coli* can cause severe respiratory and systemic infections in chickens, and it is often associated with significant economic losses in the poultry industry. Bacteriophages (phages) have been shown to be potential alternatives to the antibiotics in the treatment of bacterial infections. To accomplish that, phage particles must be able to reach and remain active in the infected organs. The present work aims at evaluating the effect of the route of administration and the dosage in the dissemination of 3 coliphages in the chicken's organs. In vivo trials were conducted by infecting chickens orally, spray, and i.m. with 10^6 , 10^7 , and 10^8 plaque-forming units/mL suspensions of 3 lytic phages: phi F78E (*Myoviridae*), phi F258E (*Siphoviridae*), and phi F61E (*Myoviridae*). Birds were killed 3, 10, and 24 h after challenge and the phage titer was measured in lungs and air sacs membranes, liver, duodenum, and spleen. When administered by spray, the

3 phages reached the respiratory tract within 3 h. Oral administration also allowed all phages to be recovered in lungs, but only phi F78E was recovered from the duodenum, the liver, and the spleen. These differences can be explained by the possible replication of phi F78E in commensal *E. coli* strains present in the chicken gut, thus leading to a higher concentration of this phage in the intestines that resulted in systemic circulation of phage with consequent phage in organs. When phages were administered i.m., they were found in all of the collected organs. Despite this better response, i.m. administration is a nonpracticable way of protecting a large number of birds in a poultry unit. In general, the results suggest that oral administration and spray allowed phages to reach and to remain active in the respiratory tract and can, therefore, be considered promising administration routes to treat respiratory *E. coli* infections in the poultry industry.

Key words: bacteriophage, *Escherichia coli* respiratory infection, dissemination, chicken

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INTRODUCTION

Colibacillosis, caused by *Escherichia coli*, is a severe infection of farmed poultry leading to high morbidity and mortality. This infection appears especially after other respiratory diseases, such as infectious bronchitis or mycoplasmosis, and is frequently associated with immunosuppressive diseases such as infectious bursal disease virus (Gumboro disease; Barnes and Gross, 1997). The increasing incidence of antibiotic resistances in *E. coli* and the restriction of the use of antibiotics in animal production (Huff et al., 2004) emphasize the importance of the evaluation of alternative antimicrobial therapies.

Once bacteriophages (phages) are obligatory and exclusive bacterial parasites, they can act as antimicro-

bial agents, a fact that has encouraged researchers to test their potential as therapeutic agents. Phages are ubiquitous in nature and are known to inhabit animals and humans. Phages penetrate the blood stream and other tissues very freely upon their administration by different routes. The potential of phages as antibacterial agents lies on their ability to destroy bacterial cells at the end of an infectious cycle. The simultaneous releasing of the progeny leads to a concentration of phages in the places where bacterial infection occurs, retaining their full biological activity (Dabrowska et al., 2005). Moreover, phage therapy only needs to decrease the number of infecting bacteria to a level that allows the host defenses to overcome the remaining infection (Levin and Bull, 2004).

However, phage therapy may fail if phages are unable to reach the target organs in the concentrations needed to trigger the infection cycle. Phages might be intolerant to the gastrointestinal tract conditions or inactivated by the immune system. Therefore, it is of ut-

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most importance to understand the dynamics of phage dissemination in the target organism to predict the success of phage therapy. In this study, the dissemination of 3 different coliphages was assessed, taking into account the phage type, the administration route, and the dosage.

MATERIALS AND METHODS

Bacteriophage Amplification

The phages used in this study were isolated from poultry sewage and screened against a pool of 148 avian pathogenic *E. coli* (APEC) strains. Phi F78E, phi F258E, and phi F61E were, respectively, lytic for 34.9, 23.5, and 45.0% of APEC strains and the 3 phages associated were active against 70.5% of the strains (Oliveira et al., 2008). The morphological characterization of the phages revealed that phi F78E and phi F61E, are 16–19-type phages, having capsids of 103×42 nm and contractile tails of 100×17 nm and both belong to *Myoviridae*. Although morphologically similar, they were shown to be genetically different (Oliveira et al., 2008). The other phage, phi F258E, is a *Siphoviridae*, T1-like phage with a circular head of 62-nm diameter and a flexible tail of 160×8 nm (Oliveira et al., 2008). Phage replication was performed by inoculating 10 mL of each phage in 100 mL of the *E. coli* hosts H561E, H816E, and H161E, respectively, for phages phi F78E, phi F61E, and phi F258E and mid-log grown in Luria-Bertani (LB) broth. This was followed by an overnight incubation at 37°C with shaking (120 rpm). This suspension was then centrifuged at $9,000 \times g$ for 10 min (rotor 19776, Sigma 3-16k, St. Louis, MO), filtered through a 0.22- μ m membrane and stored at 4°C.

The bacteriophage concentration was determined according to the plaque assay method described by Adams (1959). A volume of 100 μ L of successive dilutions of the phage suspension was mixed with 100 μ L of the respective bacteria host suspension (3- to 4-h culture) and 3 mL of LB 0.6% melted agar. This suspension was poured onto a 1.5% LB agar plate and incubated at 37°C overnight. The suspension volume was adjusted to obtain the desired phage concentration.

Experimental Design

Experiments were designed and conducted in accordance with the Federation of European Laboratory Animal Science Associations principles and the specific guidelines of animal welfare (Van Zutphen et al., 2001), based on the European Council Directive of 24 November 1986 (86/609/EEC) guidelines regarding the protection of animals used for scientific experimental purposes. According to those principles, the lowest number of animals necessary to reach the proposed goal in an in vivo experiment must be used. A total of 94 healthy 7-wk-old female growers (Rhode Island Red), obtained in a local poultry house, were housed in batteries and

subjected to a 5-d acclimation period. The chickens were monitored for the presence of commensal enterobacteria sensitive to phi F258E, phi F61E, and phi F78E. For that purpose, cloacae swabs were collected in triplicate, plated in MacConkey agar (selective and differential medium for gram-negative bacteria), and incubated at 37°C. Five to 8 pink colonies (microorganisms that ferment lactose, as *E. coli*) were selected and picked from each plate, incubated separately in 10 μ L of LB broth at 37°C for 3 to 4 h, and spread on a lawn. Then, they were tested for phage sensitivity: 20 μ L of phage was dropped on these bacteria lawns and incubated at 37°C overnight. Plates were then checked for clear zones.

Parallel trials were conducted to determine the efficacy of phage administration to chicken organism, concerning the route (oral, spray, and i.m.) and the dosage [1×10^6 , 1×10^7 , and 1×10^8 plaque-forming units (pfu)/mL]. Feed and water was available ad libitum. Groups of 3 animals were challenged with 1 mL of the phage suspension at each of the indicated phage concentration, orally with a syringe, by spray directly to the beak, or i.m. by injection in the chest muscle. One group, not challenged with the phages, was used as a control group. Birds were killed by isoflurane inhalation (IsoFlo, Abbott, Abbott Park, IL; Close et al., 1997) 3, 10, and 24 h after challenge. At necropsy, carcasses were dissected and different organs and tissues (lungs and air sac membranes, liver, duodenum, and spleen) were carefully excised, weighed, and emulsified individually in LB broth at 1:10 (wt/vol). The supernatants were decanted, centrifuged at $9,000 \times g$ for 10 min, and filtered through 0.22 μ m. The phage concentration was measured in each sample, as described above.

RESULTS

Bacteriophage Distribution in Chicken Organisms

Preliminary studies to detect host-susceptible strains to the 3 studied coliphages revealed the presence of a commensal *E. coli* strain susceptible to phi F78E.

The results of phage detection in the animal organs after oral and spray administration are presented in Tables 1 and 2, respectively. When administered by spray, all 3 phages reached the respiratory organs. The oral administration also allowed phi F61E and phi F258E to reach the lungs and the air sac membranes, being recovered from these organs at least at 10 h from challenge. The phage phi F78E remained in the same organs for the whole tested period when administered at 10^8 pfu/mL. Phi F78E was recovered from the duodenum at least 3 h after the oral administration of 10^7 and 10^8 pfu/mL suspensions; however, it was not possible to recover the other 2 phages (phi F61E and phi F258E) in this organ. Nevertheless, when spray administration was employed, all phages were found in the duodenum, and phi F78E titers were higher than the other phages

Table 1. Presence (+) or absence (–) of phages in organs and tissues after oral administration, according to the initial phage concentration and the time of slaughter (3, 10, and 24 h)¹

Phage count, pfu/mL ²	Phi F78E				Phi F258E				Phi F61E			
	A	B	C	D	A	B	C	D	A	B	C	D
10 ⁸	+++	+-	+-	++	+-	---	---	---	---	---	---	---
10 ⁷	++	+-	++	+-	++	---	---	---	+-	---	---	---
10 ⁶	---	---	---	---	---	---	---	---	---	---	---	---

¹A = lungs and air sacs; B = liver; C = duodenum; D = spleen.²pfu = plaque-forming unit.**Table 2.** Presence (+) or absence (–) of phages in organs and tissues after spray administration, according to the initial phage concentration and the time of slaughter (3, 10, and 24 h)¹

Phage count, pfu/mL ²	Phi F78E				Phi F258E				Phi F61E			
	A	B	C	D	A	B	C	D	A	B	C	D
10 ⁸	+++	---	++	---	++	---	+-	---	+-	---	+-	---
10 ⁷	++	+-	++	+-	++	---	---	---	---	---	+-	---
10 ⁶	++	+-	+-	+-	---	---	---	---	---	---	+-	---

¹A = lungs and air sacs; B = liver; C = duodenum; D = spleen.²pfu = plaque-forming unit.

(data not shown). Phi F78E could be isolated from the liver and spleen after oral and spray administration.

The presence of phages in organs after i.m. injection is shown in Table 3. All of the phages were recovered from the chicken lungs and air sac membranes, the liver, and the spleen at least 3 h after challenge (except for phi F61E, not found in the liver when administered at 10⁶ pfu/mL). Phages remained in the spleen for all of the experimental period. When injected at 10⁸ pfu/mL, all of the phages reached the intestine. Figure 1 presents the concentrations of phi F78E, phi F258E, and phi F61E recovered in the lungs and air sacs, the liver, and the spleen after i.m. injection of 1 × 10⁸ pfu. Data refer to phage enumeration at 3, 10, and 24 h of challenge.

In general, all of the phages were rescued in the spleen, the liver, and the lungs 3 h postadministration, with the maximum concentration of phi F78E and phi F258E observed in the spleen and of phi F61E in the liver.

Concerning the phage titers measured in the chicken's lungs, it was observed that, for all administered concentrations and for all of the phages, the higher-phage titers were detected 3 h after phage administration.

On the contrary, 10 h after challenge, no phage was detected in these organs.

DISCUSSION

Extraintestinal pathogenic *E. coli*, called APEC, possess specific virulence attributes commonly causing respiratory and systemic infections in poultry (chickens and turkeys), namely colibacillosis (Barnes and Gross, 1997; Zhao et al., 2005). In a phage therapy perspective, phages must be able to reach the infected organs, a fact that might be dependent on the mode of administration, the administration titer, and the phage itself. In fact, the overall results presented in this manuscript demonstrate that the phage type, the administration route, and the dose delivered were all factors in contributing to variability of bacteriophage dissemination in tissues.

Phages have been administered orally, topically, by spray, directly into body tissues, or systemically (Smith and Huggins, 1982, 1983; Berchieri et al., 1991; Sothill, 1994; Barrow et al., 1998; Sklar and Joerger, 2001; Huff et al., 2002, 2003a,b, 2004; Park and Nakai, 2003; Higgins et al., 2005). The method chosen for phage administration must guarantee the contact between phage

Table 3. Presence (+) or absence (–) of phages in organs and tissues after i.m. administration, according to the initial phage concentration and the time of slaughter (3, 10, and 24 h)

Phage count, pfu/mL ²	Phi F78E				Phi F258E				Phi F61E			
	A	B	C	D	A	B	C	D	A	B	C	D
10 ⁸	++	+++	+++	+++	+-	++	+-	+++	+-	++	+-	+++
10 ⁷	++	+++	---	+++	+-	+-	---	+++	+-	+-	+-	+++
10 ⁶	+++	++	+-	+++	+-	+-	+-	+++	+-	---	---	+++

¹A = lungs and air sacs; B = liver; C = duodenum; D = spleen.²pfu = plaque-forming unit.

particles and target pathogens. It is therefore important to ensure that, whatever route of administration, phage delivery to the infected organs will take place. In the particular case of avian respiratory infections caused by APEC, phages must be able to reach lungs and air sac membranes. On the other hand, from the practical point of view, some routes, like systemic ones, would be unfeasible, due to the large number of birds in a poultry unit. In this specific case, the most practical methods would be oral inoculation in feed or water or aerosol (spray) delivery of phages. In fact, other man-

agement practices employ one or both of these routes for suspension delivery, like most of the vaccine application (Cargill and Johnston, 2006) or some antibiotic and probiotic administration (Gillingham, 2006). Oral administration, however, could be considered an obstacle due to the potential phage inactivation during its passage through the acidic gut compartments (Ma et al., 2008).

Nevertheless, the experiments herein reported revealed the presence of phi F78E in all of the emulsified organs, after oral administration; however, this phage is susceptible to low pH values (data not shown). This apparent absence of deleterious effects of the low pH on the phages might be explained by the diluting effect of water-feed intake (ad libitum intake) on the digestive system that could raise the pH in gizzard and proventriculus (Van der Klis et al., 1993).

Another important aspect to consider is the fact that phi F78E was the phage recovered in higher amounts, compared with the other 3 phages, after oral or spray administration. This phage had a commensal *E. coli* host strain in the intestine and might have replicated there. This could be the reason why this phage reached and remained in the studied organs at higher titers for longer periods, being the only one that apparently reached the bloodstream. Based on this result, it can also be speculated that there may be an advantage of administering a nonpathogenic bacterial host together with the phage to ensure its amplification in the gut.

The ability of this phage to infect commensal *E. coli* strains can be advantageous in a phage therapy context because high internal titers of the phage are obtained. On the other hand, by infecting commensal strains, the phage might impair the flora equilibrium.

The presence of phages phi F258E and phi F61E in the respiratory tract after oral administration cannot be explained by their penetration into the bloodstream through the intestinal mucosa after reaching the duodenum because they were not rescued by liver or spleen filters against foreign organisms that enter the bloodstream. Thus, phages might have reached chickens' lungs and air sacs probably due to the inhalation of aerosols or suspension droplets during the administration. Relative to phi F78E, aerosols might have been formed and breathed as well from the dust of the cages, where the concentration of this particular phage should be higher (due to the presence of an intestinal host strain). Conversely to the other 2 phages, phi 78E was found in the liver and spleen when given orally to chickens, as well as in the duodenum, the segment of the small intestine with a higher absorption rate, indicating its absorption through the intestinal mucosa. Some researchers (Weber-Dabrowska et al., 1987; Dabrowska et al., 2005; Górski and Weber-Dabrowska, 2005; Górski et al., 2006) reported that orally administered phages can reach the peripheral blood and migrate to the infection sites. The phage occurrence in the blood is also supported by several authors (Merril et al., 1996;

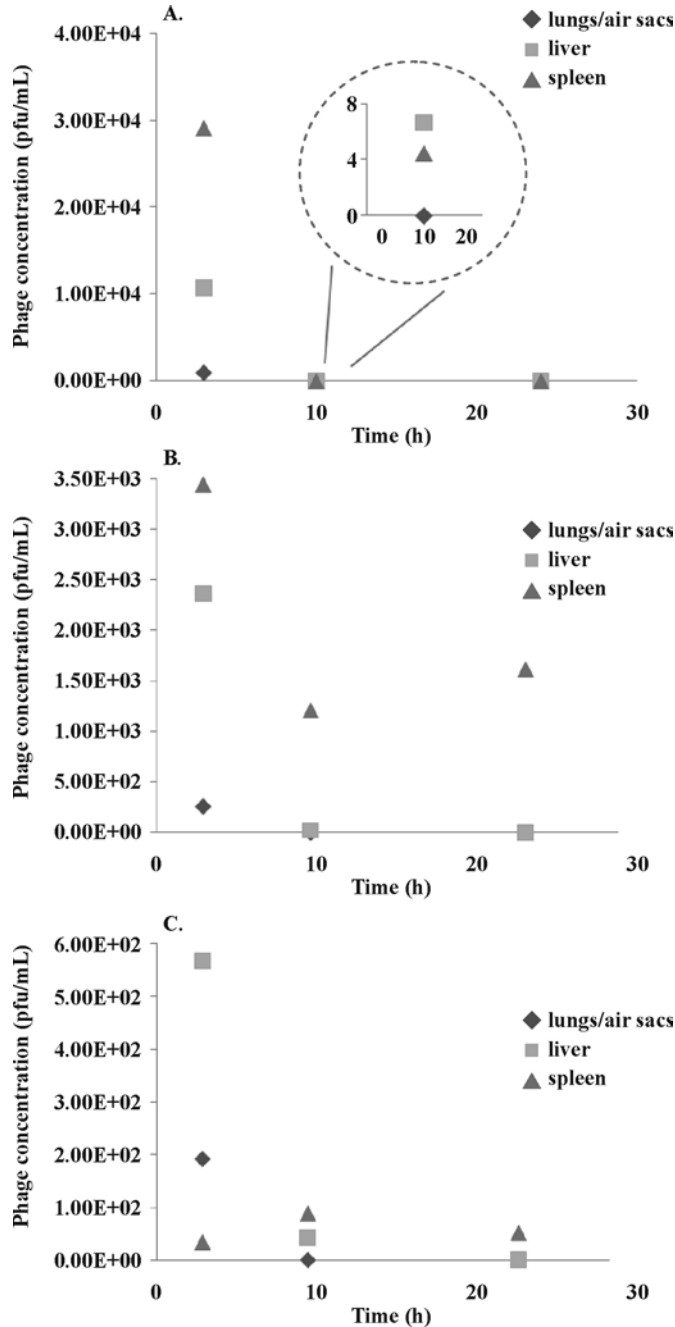


Figure 1. Concentration (pfu/mL) of phi F78E (A), phi F258E (B), and phi F61E (C) found in lungs and air sacs, liver, and spleen after 3, 10, and 24 h of the i.m. administration of 1×10^8 pfu/mL. The inset in panel A illustrates, in an amplified scale, the values of phage concentration in organs at 10 h postadministration.

Dabrowska et al., 2005; Górski and Weber-Dabrowska, 2005).

Spray administration allowed all phages to reach the respiratory tract. This may be a promising route of administration allowing phages to reside in the tissues and membranes where the pathogenic bacteria are located. Huff et al. (2003b) also reported the presence of phages in the respiratory tract after aerosol administration. The fact that with this route of administration phages reached the chicken duodenum is probably due to the spray swallowing. This route allowed phi F78E to circulate in the bloodstream, reaching all organs. Three hours after challenging the chickens i.m., it was possible to find the 3 phages in all organs including lungs and air sacs. This is an important indicator for therapy, once phages can rapidly reach the target organs of infection for pathogenic *E. coli*. However, these results indicate that although phages rapidly disseminated in the animal organs (at least 3 h after challenge) reaching the infected tissues, they were quickly cleared by the chicken organism. In fact, all of the phages were cleared from lungs after 10 h. So, for practical purposes, it can be hypothesized that in this particular case, phage therapy of respiratory infections is only efficient immediately after phage administration and the fact that phages would not confer protection against *E. coli* after 10 h might compromise their use as prophylactic agents.

Whatever the route of administration, as expected, the phage dosage seemed also to be an important factor for phage therapy in vivo efficiency. Results suggest that the initial concentration of phages administered i.m. was directly proportional to the quantity of phages that reached the potentially affected organs (data not shown). A dose-dependence effect was reported by several authors in phage efficacy studies and modeling (Payne and Jansen, 2001; Biswas et al., 2002; Weld et al., 2004).

Summarizing, phage dissemination into the chicken organs is highly dependent on the dosage and route of administration. The presence of commensal bacteria might also play an important role in phage spreading. Spray and oral phage administration enables phages to reach the chicken respiratory tract and therefore can be considered important administration routes to control *E. coli* respiratory infections.

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